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The F-box protein FBXO45 promotes the proteasome-dependent degradation of p73

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Abstract

The transcription factor p73, a member of the p53 family, mediates cell-cycle arrest and apoptosis in response to DNA damage-induced cellular stress, acting thus as a proapoptotic gene. Similar to p53, p73 activity is regulated by post-translational modification, including phosphorylation, acetylation and ubiquitylation. In *C. elegans*, the F-box protein FSN-1 controls germline apoptosis by regulating CEP-1, the single ancestral p53 family member. Here we report that FBXO45, the human ortholog of FSN-1, binds specifically to p73 triggering its proteasome-dependent degradation. Importantly, SCF^{FBXO45} ubiquitylates p73 both *in vivo* and *in vitro*. Moreover, siRNA-mediated depletion of FBXO45 stabilizes p73 and concomitantly induces cell death in a p53-independent manner. All together, these results show that the orphan F-box protein FBXO45 regulates the stability of p73, highlighting a conserved pathway evolved from nematode to human by which the p53 members are regulated by an SCF-dependent mechanism.

Keywords

ubiquitin; F-box proteins; p73; degradation; proteasome

Introduction

The covalent attachment of an ubiquitin chain to a target protein occurs through sequential steps catalysed by three different enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin ligase (E3) enzymes (Hershko, 2005).

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Conflict of interest

The authors declare no conflict of interest.

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A large number of E3s have been identified so far, reflecting their crucial role to recognize and physically bind to specific substrates (Hershko, 2005; Petroski and Deshaies, 2005; Bernassola *et al.*, 2008). The SCF complexes are the most characterized class of RING-finger-type E3s (Cardozo and Pagano, 2004). They are composed of four subunits: Skp1, Cul1, RBX1 (also known as ROC1) and a F-box protein. Cul1 is a scaffold protein that interacts at the amino terminus with the adaptor subunit Skp1 and at the carboxyl terminus with the RING-finger protein ROC1 that accounts for the binding to specific E2-conjugating enzymes. In humans, sixty-nine SCF ligases have been identified to date, each characterized by a different F-box protein subunit that provides specificity by recognizing different substrates (Cenciarelli *et al.*, 1999; Winston *et al.*, 1999). Notably, only nine out of sixty-nine human SCF ubiquitin ligases have well-established substrates, many of which are involved in cell-cycle control, cell growth, apoptosis, DNA damage response and tumorigenesis (Dorrello *et al.*, 2006; Peschiaroli *et al.*, 2006; Frescas and Pagano, 2008).

p73 is a structural and functional homolog of the tumor suppressing transcription factor p53 (Kaghad *et al.*, 1997; Tomasini *et al.*, 2008a). Similarly to p53, p73 has three different domains: an N-terminal transactivation domain, a central DNA-binding domain and C-terminal oligomerization domain (Levrero *et al.*, 1999; Melino, 2003). However, p73 displays a higher level of structural complexity than p53, existing as several distinct protein isoforms resulting from alternative splicing, alternative promoter usage or alternative initiation of translation (De Laurenzi *et al.*, 1998, 1999). In particular, the usage of the alternative internal promoter gives rise to N-terminally truncated proteins (ΔN isoforms), which lack the transactivation domain and, as a result, act as dominant negative inhibitors of p53 and TAp73 functions (Grob *et al.*, 2001).

On the basis of their structural homology, p73 and p53 should exert similar biological outcome. In fact, ectopic TAp73 is able to bind p53 responsive element and to mediate cell-cycle arrest and apoptosis in response to DNA damage-induced cellular stress (Bergamaschi *et al.*, 2003; Melino *et al.*, 2004; Ramadan *et al.*, 2005), acting thus as a proapoptotic factor. On the contrary, ΔNp73 proteins promote cell survival and exhibit oncogenic properties. Specifically, ΔNp73 has been shown to induce immortalization of primary cells, cooperate with oncogenic *Ras* in cellular transformation and promote tumor growth in nude mice (Petrenko *et al.*, 2003).

Although the role of p73 during tumorigenesis *in vivo* has been controversial (Yang *et al.*, 2000; Melino *et al.*, 2002; Flores *et al.*, 2005; Perez-Losada *et al.*, 2005), probably due to the pro- and antiapoptotic function elicited by the TAp73 and ΔNp73 isoforms, respectively, a recent paper has established a tumor suppressor function for the TAp73 (Tomasini *et al.*, 2008a). Indeed, $TAp73^{-/-}$ mice are tumor prone, sensitive to carcinogens and exhibit genomic instability (Tomasini *et al.*, 2008a, b).

TAp73 protein levels are induced by a wide variety of chemotherapeutic agents in a manner distinct from p53 (Agami *et al.*, 1999; Gong *et al.*, 1999; Yuan *et al.*, 1999), while ΔNp73 is rapidly degraded in response to cellular stress (Maisse *et al.*, 2004). As a result of the opposite activities exerted by TAp73 and ΔNp73 proteins, the balance between cell death and survival, particularly in cells harboring p53 mutations will crucially depend on the relative proportions of the two isoforms.

Similar to p53, p73 expression is maintained at low levels in mammalian cells, and its cellular induction and activation is controlled at the post-translational level, mainly by ubiquitin-dependent mechanism (Bernassola *et al.*, 2004; Maisse *et al.*, 2004; Watson and Irwin, 2006). Different E3s have been shown to regulate the stability of p73. Our group has previously demonstrated that the HECT-type E3 Itch controls the proteasome-dependent

degradation of p73, maintaining its protein levels low in unstressed conditions (Rossi *et al.*, 2005). Interestingly, in *C. elegans* the F-box protein FSN-1 controls the activity of CEP-1, the single ancestral p53 family member, thus regulating germline apoptosis (Gao *et al.*, 2008). This prompted us to investigate the possibility that the human ortholog of FSN-1, the F-box protein FBXO45, also played a role in regulating p73. Indeed, here we report data demonstrating that FBXO45 specifically binds to and promotes p73 ubiquitylation, thus triggering its proteasomal degradation. Importantly, siRNA-mediated depletion of FBXO45 induces stabilization of p73 and enhances the cell death induced by chemotherapic agents. Moreover, we show that, similarly to Itch, FBXO45 is downregulated in response to DNA damage and its decrease is essential to allow p73 levels to rise.

Results

Cul1-dependent regulation of p73 protein level

To investigate the putative role of a Cullin-based E3 ligase in controlling p73 protein levels, we took advantage of a CHO-derived ts41 cell line harboring a temperature-sensitive mutation in the APP-BP1 gene encoding the Nedd8 E1-activating enzyme (Handeli and Weintraub, 1992). Nedd8 is an ubiquitin-like protein, whose covalent attachment to cullins is required for the activity of Cullin-based E3 ligases (Morimoto *et al.*, 2003; Saha and Deshaies, 2008). We transfected HA-tagged p73 (HA-TAp73) into ts41 cells and analysed by immunoblot (IB) p73 levels either at permissive (34 °C) or not permissive (40 °C) temperature. At 40 °C the neddylation pathway is inhibited, as shown by the lack of the nedd8 modification of Cul2 (Figure 1a). In concomitance with the inactivation of the Nedd8-conjugation pathway, we observed an increase in p73 protein levels (Figure 1a). This effect is specific for p73 as the other members of the p53 family, p63 and p53, are not regulated in the same manner (Figure 1b).

In humans there are five different cullin proteins, all of them requiring the Nedd8 modification to exert their function (Hori *et al.*, 1999; Osaka *et al.*, 2000; Parry and Estelle, 2004). We investigated which cullin proteins account for the increase of the p73 level observed in ts41 cells. We co-transfected HA-TAp73 with different dominant negative mutants of cullins and then analysed p73 levels by IB. Expression of Cul1 dominant negative mutant, but not Cul2, Cul3, Cul4 and Cul5 dominant negative mutants, increased p73 levels (Figure 1c) to an extent similar to that observed in the ts41 cell line at not permissive temperature, suggesting that a Cul1-associated activity is required for p73 protein stability.

Cul1 is the scaffold protein of the SCF complexes and many substrates of this class of E3s were found to be associated with Cul1 (Donzelli *et al.*, 2002; Peschiaroli *et al.*, 2006). Therefore, we tested whether a complex between TAp73 and Cul1 can be observed *in vivo*. We performed an *in vivo* co-immunoprecipitation experiments and found that endogenous Cul1 indeed binds Flag-tagged TAp73 (Figure 1d). All these results imply a role for an SCF complex in the regulation of p73 protein level.

The F-box protein FBXO45 binds specifically to p73

In *C. elegans* the F-box protein FSN-1 controls germline apoptosis by regulating the p53 ortholog, CEP-1 (Gao *et al.*, 2008). Among the 520 predicted F-box proteins of *C.elegans*, FSN-1 is among the few to be conserved through evolution and its human ortholog is FBXO45 (Kipreos and Pagano, 2000; Thomas, 2006). This has prompted us to investigate whether FBXO45 could interact with p73. To this aim, we screened different human F-box proteins for their ability to bind to p73. Flag-tagged versions of F-box proteins were co-transfected with HA-tagged p73 into HEK293T cells (adding the proteasome inhibitor

MG132 for 6h before harvesting the cells) and then immunoprecipitated to evaluate their interaction with p73. We found that both wild-type FBXO45 and its F-box deleted mutant (ΔF-FBXO45) interacted with p73 (Figure 2a), whereas related F-box proteins such as FBXO11, FBXO4 and FBXO28 did not. Similarly, various additional F-box proteins (Skp2, βTrcp, FBXW7 and FBXO9) did not co-immunoprecipitate p73 either (Figure 2a and data not shown). Importantly, FBXO45 bound to both TAp73 and ΔNp73 isoforms, but not to p53 (Figure 2b), indicating the specificity of the interaction between FBXO45 and p73. To determine the region of p73 responsible for the binding to FBXO45, we generated a series of p73 deletion mutants and tested their ability to bind Flag-tagged FBXO45 (Figure 2c). A N-terminal p73 deletion mutant containing the oligomerization domain and the SAM domains were able to co-immunoprecipitate with FBXO45, whereas the transactivation and the DNA-binding domain localized in the N terminus did not. The p73 deletion mutant containing only the oligomerization domain did not interact with FBXO45, suggesting that the SAM domain of p73 is responsible for the binding to FBXO45 (Figure 2c).

At a structural level, human FBXO45 is characterized by an N-terminal F-box domain (spanning from amminoacids 39–82) and a SPRY domain (spanning amino acids 152–284) (Figure 2d). We investigated also which domain of FBXO45 is responsible for binding to p73. The SPRY domain of FBXO45 was still able to interact with p73 (Figure 2d), while a region containing just the F-box domain did not bind to TAp73, indicating that the SPRY domain of FBXO45 mediates the interaction with p73.

FBXO45 induces the proteasome-dependent degradation of p73

It is well established that the F-box domain accounts for the binding to the adaptor protein Skp1, whereas the other domains present in the F-box proteins are responsible for their interaction with substrates (Cardozo and Pagano, 2004). As expected, wild-type FBXO45 was able to interact with endogenous Skp1, whereas the ΔF-FBXO45 mutant did not, suggesting that the F-box domain of FBXO45 is functionally important to form an SCF complex *in vivo* (see Supplementary Figure S1).

Since the SCF complexes generally regulate the proteasome-dependent degradation of target proteins, we studied the effect of overexpressing FBXO45 on p73 levels. HA-TAp73 was co-transfected into neuroblasoma SH-SY5Y cells in the presence of either wild-type Flag-tagged FBXO45 or ΔF-FBXO45. As shown in Figure 3a, TAp73 protein levels were efficiently downregulated by the coexpression of wild-type FBXO45, both under unstressed conditions and after DNA damage. FBXO45 expression was also able to negatively modulate the expression of ΔNp73 (Figure 3b). Importantly, the ΔF-FBXO45 mutant, which was still able to bind p73 (see Figures 2a and d), was not capable to negatively modulate both TAp73 and ΔNp73 levels (Figures 3a and b and Supplementary Figure S2), confirming the requirement of an intact SCF^{FBXO45} complex for regulating p73 protein levels. To assess whether the FBXO45-mediated dowregulation of p73 is due to proteasome-dependent degradation, we treated SH-SY5Y cells expressing both wild-type FBXO45 and p73 with the proteasome inhibitor MG132. As shown in Figure 3c, exposure to MG132 totally rescued FBXO45-dependent downregulation of p73, demonstrating that FBXO45 controls the proteasome-dependent degradation of p73 *in vivo*.

SCF^{FBXO45} ubiquitylates p73 *in vivo* and *in vitro*

Because a major function of F-box proteins involves the ubiquitylation of their target substrates, we sought to determine whether FBXO45 ubiquitylates p73 in mammal cells. To this aim, we transfected the constructs expressing myc-tagged FBXO45, Flag-tagged TAp73 and HA-tagged ubiquitin into SH-SY5Y cells. We then performed immunoprecipitation with an anti-FLAG antibody-conjugated M2 beads under denaturing conditions to eliminate any

p73-associated proteins through non-covalent bonds. Ubiquitin-conjugated p73 species were detected by IB with an anti-Flag antibody. As shown in Figure 4a, the expression of FBXO45 enhanced the ubiquitylation of p73 as compared with the control vector (compare lane 2 with lane 4). We also reconstituted the ubiquitylation of p73 *in vitro* by using a semipurified system. Flag-tagged wild-type FBXO45 or its mutant ΔF-FBXO45 were transfected into HEK293T cells and then purified by immunoprecipitation with anti-Flag antibody. After extensive washes, the beads containing the SCF^{FBXO45} or $SCF^{\Delta F-FBXO45}$ complexes were mixed to an *in vitro* ubiquitylation reaction containing immunopurified TAp73. FBXO45, but not its F-box deleted mutant ΔF-FBXO45, was able to promote the ubiquitylation of purified p73 (Figure 4b). A different F-box protein, FBXO11, was unable to trigger the ubiquitylation of p73, confirming the specificity of FBXO45. All these results indicate that FBXO45 promotes p73 ubiquitylation both *in vivo* and *in vitro*.

Silencing of FBXO45 induces p73 stabilization and cell death in the absence of functional p53

p73 activity controls different processes, including the apoptotic response to DNA damage and neuronal differentiation (Yuan *et al.*, 1999; De Laurenzi *et al.*, 2000). To test whether the DNA damage response regulates FBXO45 expression, we performed an RT– PCR analysis in cells treated with doxorubicin, a DNA damage-inducing agent. Figure 5a showed that FBXO45 expression was downregulated after DNA damage both in SH-5YSY cells and BT-20 breast cancer cells. On the contrary, we did not observe any significant variation of FBXO45 mRNA levels in SH-5YSY cells treated with retinoic acid to induce neuronal differentiation (data not shown). The results presented above prompted us to test whether the depletion of FBXO45 could increase p73 protein levels and thus potentiate apoptotic response to a DNA-damage agent. To this end, we used the siRNA technique to reduce the expression of FBXO45 in the BT-20 cell line, which harbors a mutation in the p53 locus, thus, avoiding p53-dependent apoptosis (Chopin *et al.*, 2002). We first tested two different siRNA oligos for their ability to downregulate FBXO45. Both oligos were able to decrease FBXO45 expression at the mRNA level and induced a concomitante accumulation of p73 (Figure 5b). Importantly, FBXO45 depletion did not affect the mRNA level of p73 (Figure 5b), indicating that it specifically controls p73 protein stability.

To determine whether FBXO45 downregulation could sensitize cells to apoptosis, we treated the FBXO45-depleted cells with doxorubicin and we measured the apoptotic index after DNA damage. In concomitance with the upregulation of p73, FBXO45-depleted cells showed a marked increase in the apoptotic index (Figure 5c), indicating thus that FBXO45 could regulate cell death in the absence of functional p53.

Discussion

The F-box proteins recruit specific substrates to the E3 ligase SCF complex, thus targeting them to proteasome-dependent degradation (Cardozo and Pagano, 2004). Despite the large number of F-box proteins, only nine human SCF ubiquitin ligases have well-established substrates, many of which are involved in cell-cycle control, apoptosis and DNA damage response (Peschiaroli *et al.*, 2006; Frescas and Pagano, 2008).

p73, a member of the p53 family, is a transcription factor controlling different biological processes, including cell death, tumorigenesis and neuronal differentiation (Melino, 2003). Similar to p53, p73 activity and protein levels are strictly controlled by post-translational modifications, including ubiquitylation. Knowledge on the mechanisms regulating p73 levels in basal conditions as well as in response to stress is essential to design new therapies that require p73 induction. Our group has previously shown that the HECT E3 ubiquitin ligase Itch is capable to polyubiquitylate p73 and induce its degradation in a proteasome-

dependent manner. In unstressed cells, Itch targets both TAp73 and δNp73 isoforms for protein ubiquitylation, thereby keeping their expression levels low under normal conditions (Rossi *et al.*, 2005). In response to DNA-damaging agents, Itch is downregulated allowing TAp73 levels to rise. Clearly, this is not the only mechanism regulating p73 stability and here we report indeed the identification of the F-box protein FBXO45 as a new player in the regulation of p73. We show that FBXO45 is able to bind to p73 promoting its ubiquitylation and its proteasome-dependent degradation. Moreover, we found that FBXO45, similar to Itch, is downregulated in response to DNA damage, allowing thus p73 levels to increase in response to stress.

p73 gene encodes for two major proteins, the proapoptotic TA and the antiapoptotic δN isoform (Levrero *et al.*, 1999). Our data indicates that exogenously expressed FBXO45 is able to bind to both TA and δN isoforms with similar affinities, suggesting that FBXO45, similar to Itch, is not able to discriminate between these two isoforms. In agreement with this, FBXO45 overexpression promotes the proteasome-dependent degradation of both TA and δN isoforms. We cannot rule out the possibility that the endogenous expression levels of the two isoforms and/or different stimuli can determine a preferential binding to FBXO45 of one isoform with respect to the other, inducing thus different biological outcomes. However, our data once again leaves us with the idea that an unidentified degradation pathway, activated in response to DNA damage, specific for δNp73, must exist.

The importance of SCF-dependent regulation of p73 is also highlighted by the fact that this mechanism is conserved through evolution. As a matter of fact a recent report has described that the worm ortholog of FBXO45, the F-box protein FSN-1, is implicated in germline apoptosis through the regulation of the apoptotic activity of CEP-1 (Gao *et al.*, 2008). Similar to its vertebrate relatives, the *C.elegans* p53-like gene *CEP-1* is the key regulator of the cell death after genotoxic stress (Derry *et al.*, 2001; Schumacher *et al.*, 2001). CEP-1 expression is induced after DNA damage and its activation controls germline apoptosis. Gao *et al*. reported that CEP-1 activity is strictly regulated by the SCF complex SCF^{FSN-1}. FSN-1-null mutants exhibit higher induction of CEP-1 levels after genotoxic stress and concomitantly a higher apoptotic index. Structurally the DNA-binding domain of CEP-1 is similar to the one of human p53, but its C terminus contains a SAM domain that resembles the C terminus of vertebrate p63 and p73 (Ou *et al.*, 2007), suggesting its similarity with the p63/p73 ancestor. Although we cannot completely rule out a possible involvement of FBXO45 in the regulation of p63 and p53 activity, our data indicate that FBXO45 controls specifically the protein degradation of p73. In fact, FBXO45 is able to interact with p73, but not with p53. Moreover, we found that the protein level of p53 and p63 are not affected by the inhibition of the neddylation pathway, suggesting that the activity of the SCF complex is not implicated in the regulation of p53/p63 stability. Accordingly, it has previously described that the neddylation pathway regulates the transcriptional activity of p53, but not its stability (Abida *et al.*, 2007).

In *C. elegans*, the genetic depletion of FSN-1 or Cul1 induces germline apoptosis through the increase of CEP-1 protein levels (Gao *et al.*, 2008). In line with these data, we showed that the siRNA-mediated depletion of FBXO45 increases p73 protein levels and sensitizes cells to DNA damage inducing apoptosis. Moreover, we found that the expression of the dominant negative mutants of Cul1, but not of Cul2, Cul3, Cul4 and Cul5 dominant negative mutants, induces p73 protein stabilization. From all these data, it seems that an SCF-dependent regulation of the p53 family members is a mechanism that has been conserved through evolution, reinforcing the important physiological role played by this pathway.

The F-box proteins generally recruit substrates in a phosphorylation-dependent manner (Skowyra *et al.*, 1997). Different kinases have been reported to be able to phosphorylate

p73. In response to DNA-damaging agents, the non-receptor tyrosine kinase c-Abl phosphorylates p73 at Tyr99 leading to an increase in p73 stability (Gong *et al.*, 1999). Moreover, p73 was found to interact with cyclin-dependent kinases such as cyclin A/cdk2 and cyclin E/cdk2, and threonine 86 of p73 could be phosphorylated by these kinases *in vitro* (Gaiddon *et al.*, 2003). More recently, the cell cycle regulated kinase Plk1 has been shown to interact with p73 and to induce its phosphorylation at threonine 27 (Koida *et al.*, 2008; Soond *et al.*, 2008). Interestingly, the Plk1-dependent phosphorylation of p73 seems to negatively regulate p73 protein stability (Soond *et al.*, 2008). However, our unpublished results suggest that Plk1 activity is not involved in FBXO45-dependent degradation of p73. It will be important in the future to determine whether the FBXO45-dependent degradation of p73 is dependent on some of the above-mentioned phosphorylation events or on another kinase activity.

In conclusion, we showed that FBXO45 binds to and stimulates the proteasome-dependent degradation of p73. As FBXO45 depletion sensitizes cell to apoptosis in a p53-null background, we elucidate a potential new mean that could be used to develop new strategies aimed to potentiate the apoptotic response of cancer cells after chemotherapy. Importantly, we undercovered a conserved mechanism by which the p53 family members are regulated in response to genotoxic stress.

Materials and methods

Cell culture, transfection conditions and reagents

Human embryonic kidney HEK293T, human epithelial carcinoma HeLa, human neuroblastoma SH-5YSY and human breast cancer cell line BT-20 were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Chinese hamster ovary Ts41 cell line was maintained at 34°C, and then shifted to non-permissive temperature (40 °C) to inhibit the neddylation pathway. Transfections were performed using the calcium phosphate method for HEK293T, Effectene (Qiagen, Hilden, Germany) for HeLa and BT-20 and Lipofectamine LTX (Invitrogen, Grand Island, NY, USA) for SH-5YSY, according to the manufacturer's instructions. Cells have been treated for 6 h with 10 μM proteasome inhibitor MG132 (Calbiochem, San Diego, CA, USA) and/or with 2 μM doxorubicin hydrochloride (Sigma, Taufkirchen, Germany) when appropriate. The following pcDNA3.1 expression constructs for the F-box proteins were kindly provided by Michele Pagano (NYU School of Medicine): myc-FBXO45, Flag-βTrCP, Flag-FBXO4, Flag-FBXO28 and Flag-Skp2. The pcDNA3.1 expression vector for wild-type Flag-tagged FBXO45 has been obtained by subcloning the FBXO45 cDNA into the pcDNA3.1 Flag-B vector. The ΔF-FBXO45 deletion mutant has been generated by PCR using the following primers: 5′-CGGGATCCATGGATTACAAG GAGGATGACGACG ATAAGTGCGCCCGCAGCCTGG CAGAAGAG-3′ and 5′-CCGCTCGAGTCATCCGTCCAAA GGTTTTCCAAGGTAAACC-3′. The ΔSPRY deletion mutant was generated by digestion with *Apa*1 restriction enzyme using pcDNA3.1 Flag wt-FBXO45 as a template followed by ligation.

Immunoblot analysis, immunoprecipitation and antibodies

Immunoblot analysis has been performed using whole-cell extracts obtained by lysing the cell pellet with Triton Buffer (50 mM Tris-Hcl, pH 7.5, 250 mM NaCl, 50 mM NaF, 1mM EDTA 1 pH 8, 0,1% Triton), supplemented with proteases and phosphatases inhibitors, and with 20 μM *N*-ethylmaleimide (Sigma) when required. Proteins were subsequently separated by SDS–polyacrylamide gel electrophoresis, blotted onto poly-vinylidene difluoride membranes (Millipore, Billerica, MA, USA) and then blocked with phosphate-buffered

saline and 0.1%Tween-20 containing 5% non-fat dry milk for 1 h at room temperature. The incubation with primary antibodies was performed for 2 h at room temperature, followed by appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After extensive washing, detection was performed with the ECL Western Blot Chemiluminescence Reagent (Perkin Elmer, Waltham, MA, USA).

Immunoprecipitation has been performed by preclearing 1 mg of whole cell lysates with protein A/G-Sepharose beads (GE Healthcare, Chalfont St. Giles, UK) for 3 h and then by incubating overnight at 4°C with the appropriate amount of antibody per sample. Finally, the immunocomplexes have been absorbed on protein A/G-Sepharose beads, washed six times in Triton lysis buffer and used for *in vitro* ubiquitylation assay or eluted by boiling in SDS loading buffer.

We used the following antibodies: mouse monoclonal anti-HA (ascites, Covance, Princeton, NJ, USA), rabbit polyclonal anti-GFP (Roche, Indianapolis, IN, USA), rabbit polyclonal anti-Cul2 (Zymed, Invitrogen), mouse monoclonal anti-β-actin (Sigma), mouse monoclonal anti-Myc (Cell Signaling, Beverly, MA, USA), rabbit polyclonal anti-Flag (Sigma), mouse monoclonal anti-Cul1 (Zymed), mouse monoclonal anti-p53 (DO-1, Santa Cruz, Santa Cruz, CA, USA), mouse monoclonal anti-p73 (Imgenex, San Diego, CA, USA) and rabbit polyclonal anti-p73 (Rossi *et al.*, 2005).

Ubiquitylation assays

In vivo ubiquitylation assay was performed using SH-5YSY neuroblastoma cells, transiently transfected with cDNAs encoding human Flag-tagged p73 (Flag-p73), myc-tagged FBXO45 (myc-FBXO45) and HA-tagged ubiquitin (HA-Ub) and treated with MG132 before collecting. Cells were lysed in denaturing condition (0.15 mM NaCl/0.05 mM Tris • HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) in the presence of 20 M *N*-ethylmaleimide (Sigma), and Flag-p73 has been immunoprecipited as described above. Polyubiquitylated p73 species were detected using anti-Flag antibody.

In vitro ubiquitylation assay was carried out using SCF^{FBXO45} complexes immunopurified from HEK393T cells transfected with His-tagged Skip and Flag-tagged FBXO45 using the method described above. The beads containing the SCF^{FBXO45} immunocomplexes were washed twice with equilibration buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.01% Triton and 10% glycerol) and then added to the following reaction: 30 ng of E1, 0.5g of UbcH3, 0.5g of UbcH5, 5g of ubiquitin in the ubiquitylation buffer (2.5 mM Tris-HCl, 0.7 mM dithiothreitol, 4 mM ATP, 10 mM $MgCl_2$, 0.1 mM ubiquitin aldehyde) in a final volume of 30 l. The HA-tagged p73 (HA-p73) protein used as substrate for the reaction has been purified by immunoprecipitation with the hemagglutinin-antibody and then eluted with the hemagglutinin peptide (1 mg/ml). The assays have been carried out for 90 min at 30 °C, then the immunocomplexes were eluted by boiling in SDS loading buffer and loaded in SDS–polyacrylamide gel electrophoresis.

siRNA-mediated silencing of FBXO45 and detection of apoptosis

SH-5YSY and BT-20 cells were seeded at a density of 1.4 × 105 cells per well in a six-well plate and transfected with oligos twice (at 24 and 48 h after plating) using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Two different 21-nucleotide RNA oligos were used, targeting the following FBXO45 cDNA sequences: 5′-CAGACGTTA CTATTATCCCTA-3′ and 5′-CTGGTGGACAATAATCTA CTA-3′. A 21-nt siRNA duplex corresponding to a non-relevant gene (scramble) was used as control. Cells were collected after 48 h and lysates were subjected to western blot. To evaluate the efficiency of silencing, we measured the FBXO45 mRNA level by reverse transcription–PCR using total RNA

extracted by FBXO45-depleted cells. Alternatively, after siRNA transfection, we transfected the siRNA expressing cells with vector encoding Flag-tagged FBXO45. After 24 h, cells were lysed and total cell extracts were analysed by IB using the Flag antibody.

Quantification of sub-G1 population was performed by FACS analysis of propidium iodide-staining nuclei (Nicoletti *et al.*, 1991), carried out in an FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) using the CELL-Quest software system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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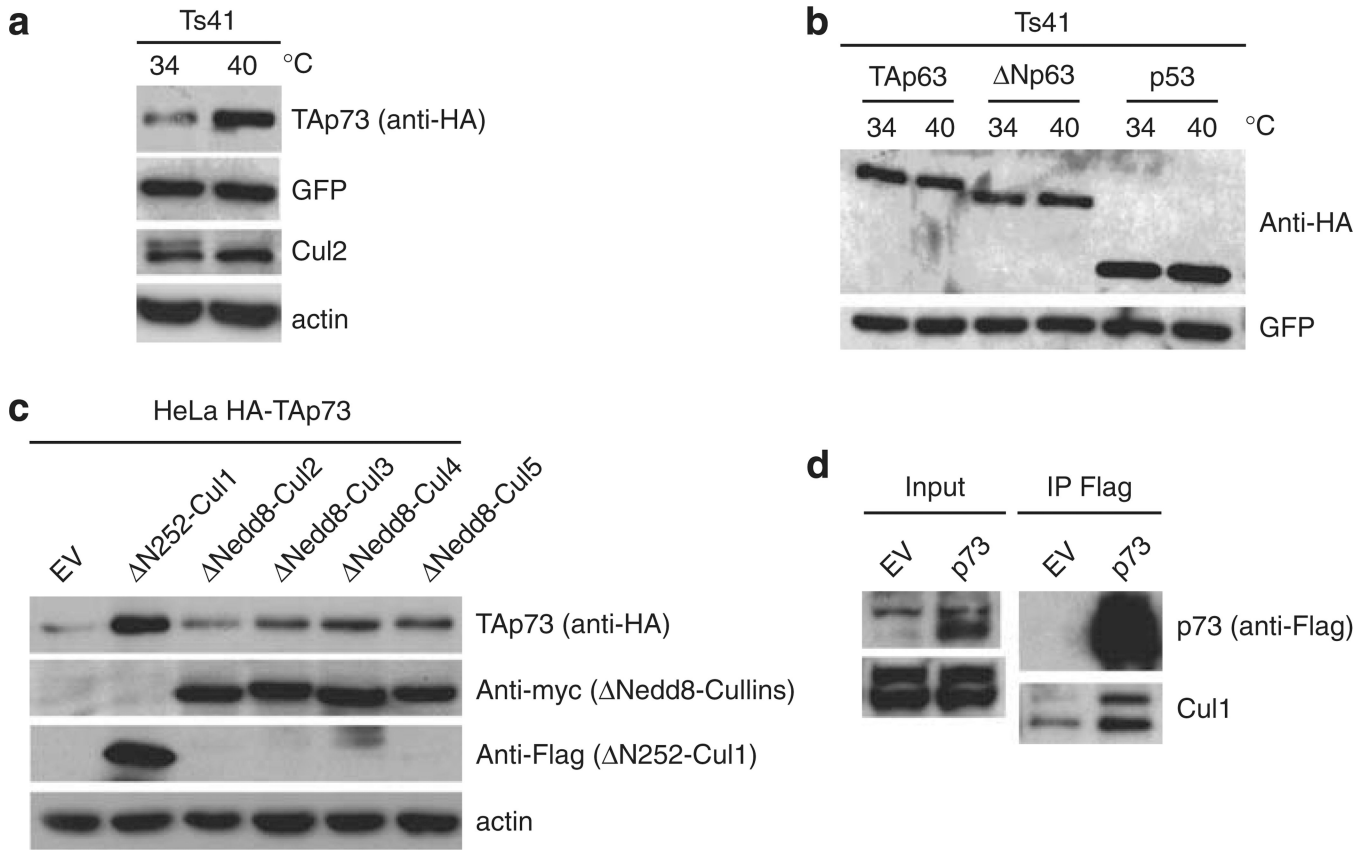

Figure 1.

Cul1-associated activity regulates p73 protein levels. **(a)**, ts41 cells were co-transfected at permissive temperature (34 °C) with HA-tagged TAp73 (HA-TAp73) and green fluorescent protein (GFP). Twenty-four hours after transfection, cells were either left at the permissive temperature or shifted to non-permissive temperature (40 °C) for 24 h. Cell lysates were immunoblotted with anti-HA and anti-actin antibodies. Blots were re-probed with antibodies against Cul-2 and GFP, as a control of impaired neddylation pathway and transfection efficiency, respectively. **(b)**, ts41 cells were co-transfected with either HA-TAp63, HA-ΔNp63, or HA-p53 together with GFP. Cells were treated as in A, and cell lysates were analysed by immunoblotting (IB) with antibodies against HA and GFP. **(c)**, HeLa cells were transfected with a plasmid coding HA-TAp73 in the absence or in the presence of the Flag-tagged ΔN252Cul1 mutant, and different myc-tagged neddylation site mutants of Cul2, Cul3, Cul4 and Cul5. Cellular extracts were analysed by IB using anti-HA, anti-Flag and anti-myc antibodies. Blots were re-probed with anti-actin antibody as loading control. **(d)** HEK293T cells were transfected with Flag-tagged TAp73 and cell extracts were immunoprecipitated with anti-Flag antibody and analysed with anti-Flag and anti-Cul1 antibodies.

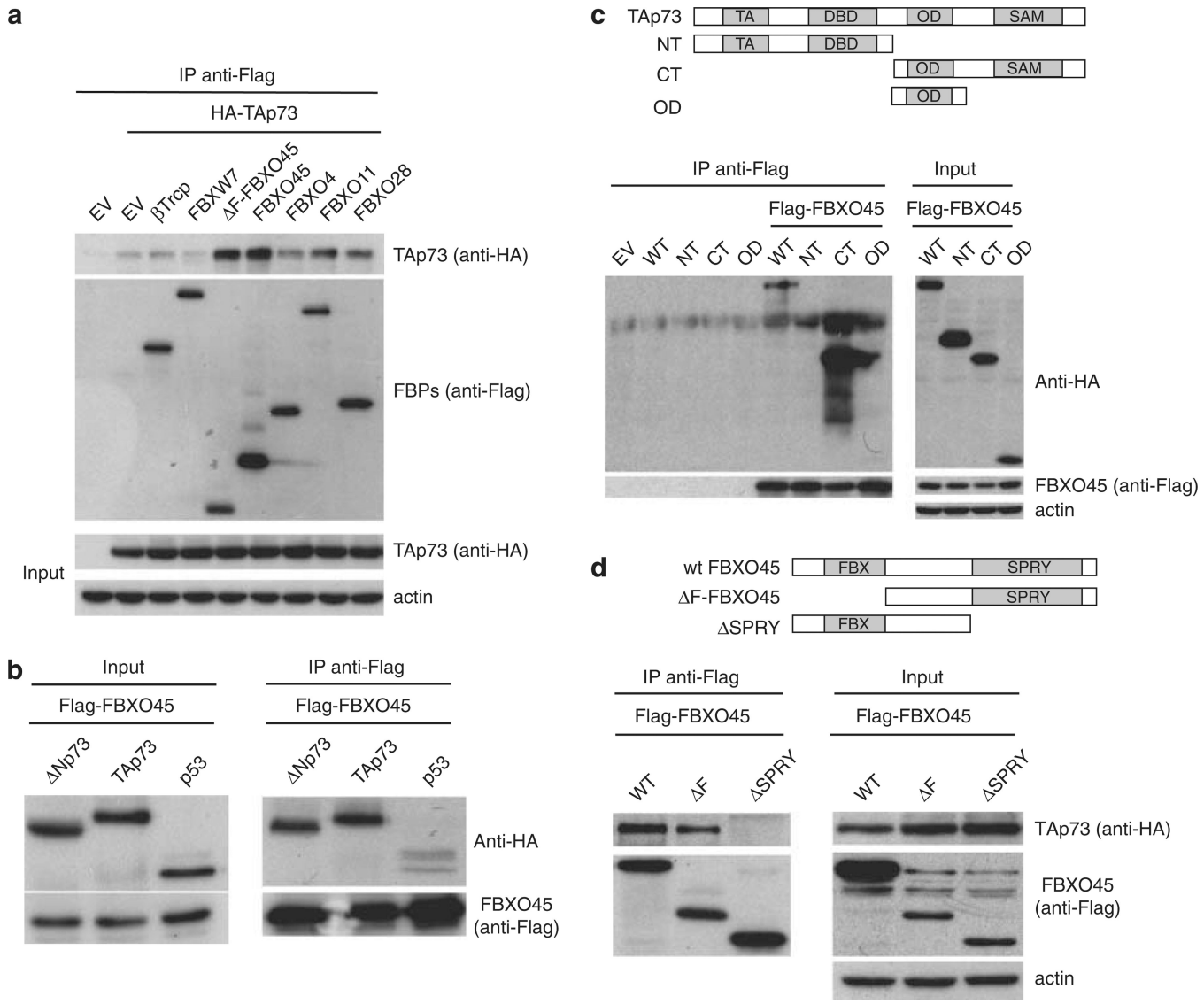

Figure 2.

FBXO45 interacts specifically with p73, both TA and ΔN isoforms. **(a)**, HEK293T cells were co-transfected with the indicated Flag tagged F-box protein constructs (FBPs) and HA-TAp73. During the last 6 h before harvesting, cells were treated with 10 μM of the proteasome inhibitor MG132. Exogenous proteins were immunoprecipitated (IP) from cell extracts with anti-Flag resin and immunocomplexes were probed with antibodies to the indicated proteins. (EV=empty vector). **(b)**, HEK293T cells were co-transfected with Flag-tagged FBXO45 (Flag-FBXO45) together with either HA-TAp73, HA-ΔNp73 or HA-p53. Cells extracts were IP using an anti-Flag antibody and immunocomplexes were probed with antibodies to the indicated proteins. **(c)**, HEK293T cells were co-transfected with the indicated HA-tagged p73 deletion mutants and Flag-tagged FBXO45. During the last 6 h before harvesting, cells were treated with 10 μM of the proteasome inhibitor MG132. Exogenous proteins were IP from cell extracts with anti-Flag resin and immunocomplexes were probed with antibodies to the indicated proteins. (EV, empty vector). **(d)**, HEK293T cells were co-transfected with the indicated Flag-tagged FBXO45 deletion mutants and HA-tagged TAp73. During the last 6 h before harvesting, cells were treated with 10 μM of the

proteasome inhibitor MG132. Exogenous proteins were IP from cell extracts with anti-Flag resin and immunocomplexes were probed with antibodies to the indicated proteins.

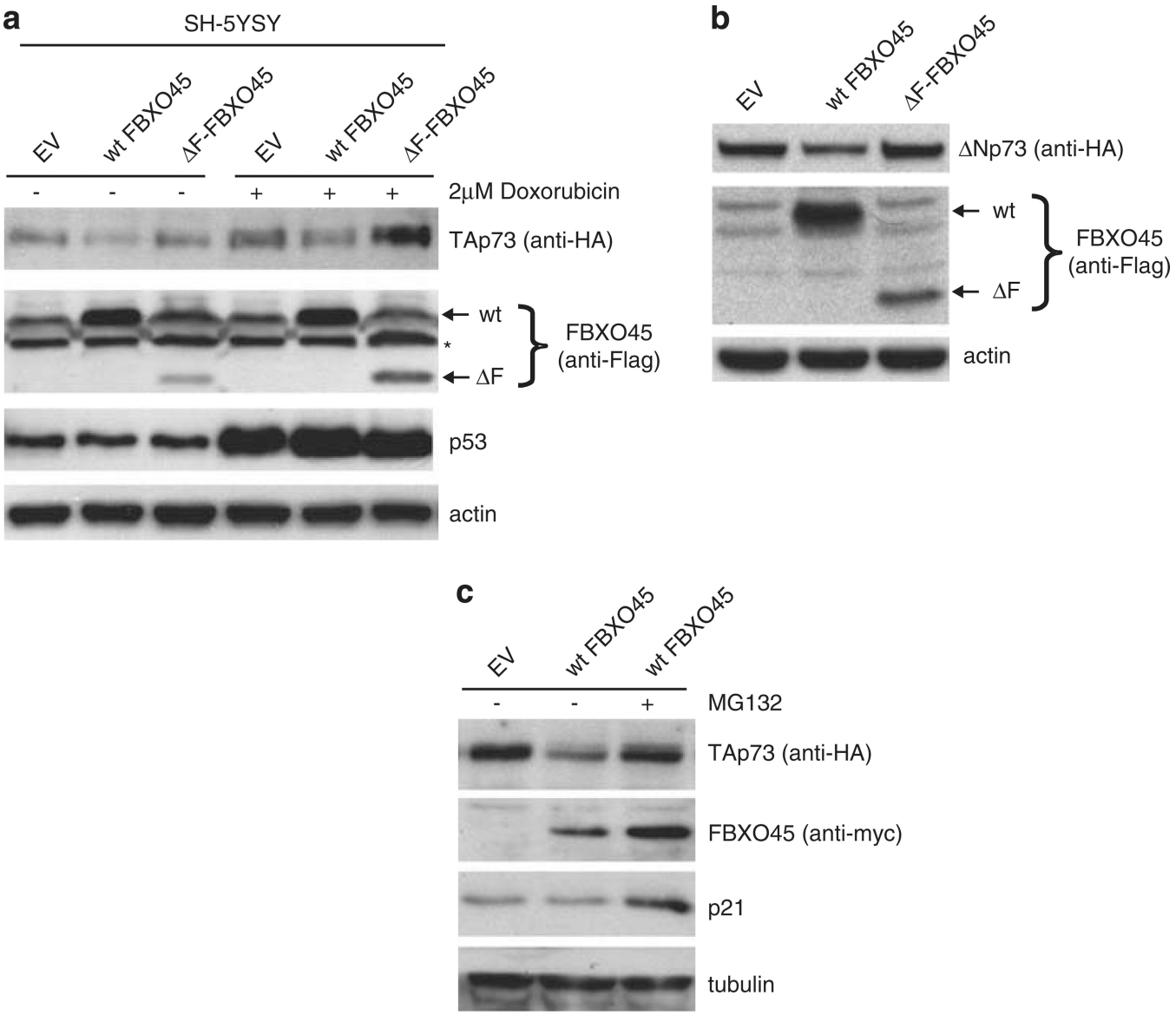

Figure 3.

FBXO45 promotes the proteasome-dependent degradation of p73. **(a)** SH-5YSY neuroblastoma cells were co-transfected with HA-TAp73 together with either wild-type Flag-FBXO45 or its mutant Flag-ΔF-FBXO45. Twenty-four hours after transfection, cells were either left untreated or treated with doxorubicin (2 μM) for 16 h. Cellular extracts were analysed by immunoblot (IB) using anti-HA and anti-Flag antibodies. Blots were re-probed with anti-actin as loading control. (EV =empty vector). Asteriks represents a nonspecific band. **(b)** SH-5YSY neuroblastoma cells were co-transfected with HA-ΔNp73 together with either wild-type Flag-FBXO45 or its mutant Flag-ΔF-FBXO45. Cellular extracts were analysed by IB using anti-HA and anti-Flag antibodies. Blots were re-probed with anti-actin as loading control. **(c)** The experiment was performed as in **(a)** except that 10 μM of MG132 was added where indicated.

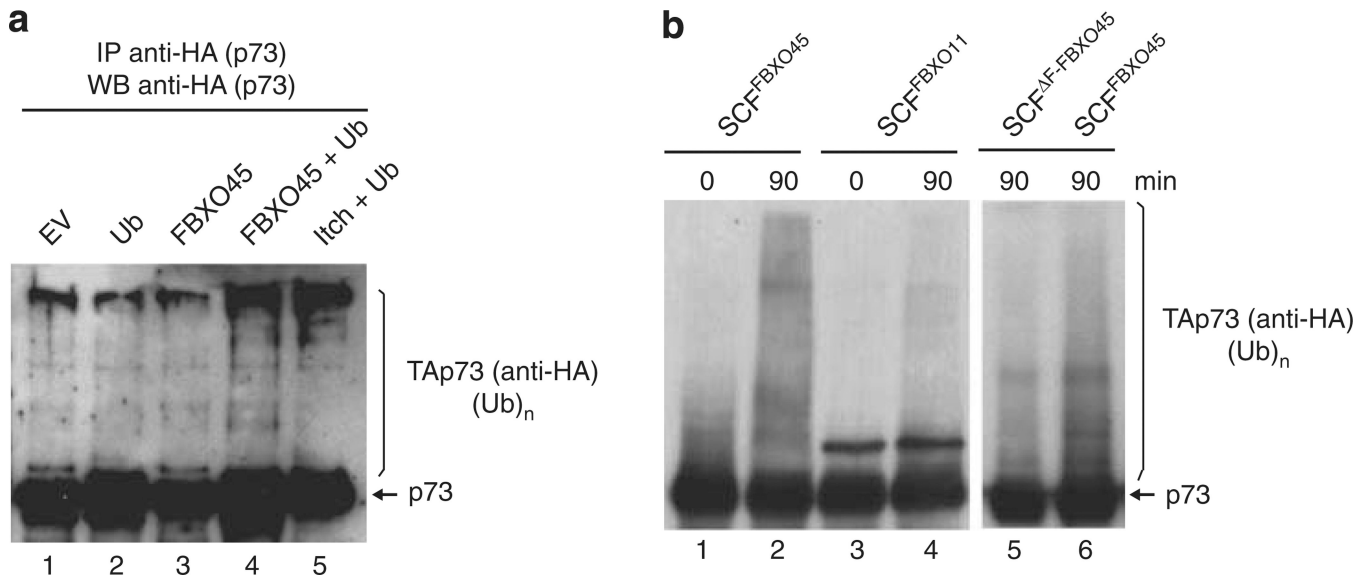

Figure 4.

FBXO45 stimulates the ubiquitylation of p73 both *in vivo* and *in vitro*. **(a)** *In vivo* ubiquitin ligation assay of TAp73 was conducted as described in Bloom and Pagano, 2005. Briefly, SH-5YSY neuroblastoma cells were co-transfected with HA-TAp73 alone or in the presence of the following proteins: empty vector (EV, lane 1), myc-tagged ubiquitin (lane 2), Flag-FBXO45 (lane 3), Flag-FBXO45 and myc-ubiquitin (lane 4), Flag-Itch and myc-ubiquitin (lane 5). After 24 h, cells were treated with 10 μM of proteasome inhibitor MG132 for 6 h and then collected. Cell extracts were subjected to immunoprecipitation before denaturation of protein samples. The immunoicomplexes were resolved in SDS–PAGE and analysed by IB using the antibodies for the indicated proteins. **(b)** *In vitro* ubiquitin ligation assay of immunopurified HA-TAp73 was conducted in the presence or absence of the following proteins: immunopurified SCF^{FBXO45}, immunopurified SCF^{FBXO11}, immunopurified $SCF^{\Delta F-FBXO45}$. Samples were incubated at 30 °C for 90 min except that in lanes 1 and 3 that were added to sample buffer. The bracket on the right side of the panel marks a ladder of bands corresponding to polyubiquitylated p73.

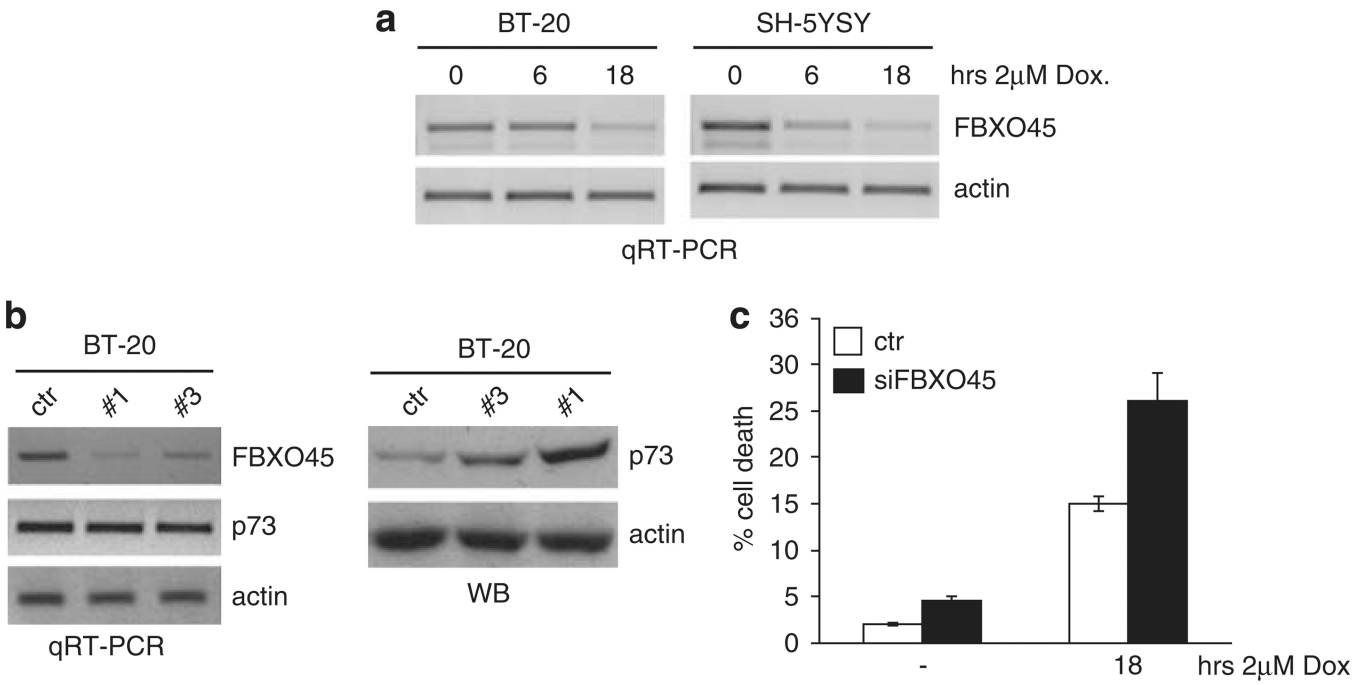

Figure 5.

FBXO45 expression is downregulated after DNA damage and its depletion by siRNA sensitizes BT-20 breast cancer cell line to doxorubicin-induced cell death. **(a)** BT-20 breast cancer cell line and SH-5YSY neuroblastoma cells were either left untreated or treated with doxorubicin (2 μM) for 6 and 18 h. Total RNA was extracted from the cells and quantitative RT–PCR (qRT–PCR) was performed using specific primer for FBXO45 and actin. **(b)** BT-20 cells were transfected twice with siRNA molecules to a non-relevant mRNA (ctr) or to two different oligos specific for FBXO45 (#1 and #3). Forty hours after transfection, total RNA was isolated and qRT–PCR was performed as described in **(a)**. Concomitantly cell extracts were subjected to immunoblot using the indicated antibody. **(c)** BT-20 cells transfected with siRNA as in **(c)**, were either left untreated or treated with doxorubicin (2 μM) for 18 h. The percentage of sub-G1 cells was measured by FACS analysis. A representative results is shown (mean ± s.d., $n = 3$).